

1 **Characterization of Disease Course after Intramuscular or Intranasal Exposure to Sin**
2 **Nombre virus in Immunosuppressed Syrian Hamsters**

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20 **Summary Statement:** This report describes the characterization of a small animal model that
21 can be utilized for the development of medical countermeasures to hantavirus disease.

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23

24 **ABSTRACT**

25 Syrian hamsters exposed to Sin Nombre virus (SNV) become infected, but do not
26 develop disease. In contrast, hamsters immunosuppressed with dexamethasone (Dex) and
27 cyclophosphamide (CyP) and infected with SNV develop lethal disease resembling hantavirus
28 pulmonary syndrome (HPS) in humans. Here, we provide a detailed analysis regarding the
29 kinetics of virus dissemination after both intramuscular and intranasal challenge. Our findings
30 revealed a ~8 day lag in the spread of virus to the lungs, kidney, liver, spleen, and heart that
31 correlates with a delayed mean day-to-death observed when immunosuppressed hamsters are
32 infected by the intranasal versus the intramuscular route. The length of immunosuppression with
33 Dex and CyP required for lethal disease for intramuscular challenge was 10 days; however 19
34 days of treatment were required in the intranasal model. Additionally, we investigated if the
35 steroid-sparing potential of an alternative immunosuppressive drug, mycophenolate mofetil
36 (MMF) could replace the combination of Dex and CyP to produce lethal disease. However,
37 MMF treatment did not increase viral replication in the lung unless it was combined with Dex.
38 Furthermore, treatment of SNV-infected hamsters with MMF or Dex/MMF did not result in
39 comparable lethality to hamsters administered Dex/CyP. Taken together, these experiments
40 further refine the SNV disease model in hamsters for future use in the evaluation of medical
41 countermeasures.

42 **INTRODUCTION**

43 Sin Nombre virus (SNV, genus *Hantavirus*, family *Bunaviridae*) is the predominant
44 hantavirus in North America, and has been associated with sporadic outbreaks of lethal human
45 disease, including the 1993 outbreak in the Four Corners region of the United States (Duchin et
46 al., 1994) and the more recent 2012 outbreak in Yosemite National Park (Centers for Disease and
47 Prevention, 2012). SNV is an etiological agent of hantavirus pulmonary syndrome (HPS), that is
48 characterized in humans by leukocytosis, thrombocytopenia, and the rapid onset of acute
49 respiratory distress (Duchin et al., 1994; Manigold and Vial, 2014). Although HPS outbreaks are
50 isolated and sporadic, unpredictable increases in the rodent vector populations can lead to
51 significant increases in human cases (Oliveira et al., 2016). In addition, the high case fatality rate
52 of approximately 35% (Jonsson et al., 2010) and the absence of specific drugs or vaccines to
53 treat or prevent HPS makes medical countermeasure development imperative. Well-

54 characterized animal models for the study of SNV pathogenesis will facilitate the development
55 of medical countermeasures.

56 Currently, there are two SNV animal models that recapitulate human HPS disease. These
57 are a small animal model utilizing immunosuppressed Syrian hamsters (Brocato et al., 2014) and
58 a nonhuman primate model using virus isolated directly from the rodent host (Safronet et al.,
59 2014). The use of these two animal models may represent a pathway to licensure for drugs or
60 biological products using the “Animal Rule” (21 CFR 314.600 and 21 CFR 601.90), respectively
61 (Snoy, 2010). Therefore, continued characterization of these models enhances their potential
62 suitability for use in drug or biological efficacy testing. In this report, we expand on our previous
63 data set by investigating the kinetics of SNV in immunosuppressed hamsters and refine the
64 length of immunosuppression required for disease.

65 Concerns over toxicity observed in lighter hamsters treated with Dex and CyP, along with
66 an effort to reduce daily injections required with Dex/CyP treatment, led us to search for
67 alternative immunosuppression methods. MMF (Cellcept, Genentech, Inc.), administered orally,
68 is hydrolyzed to mycophenolic acid, an uncompetitive inhibitor of inosine monophosphate
69 dehydrogenase that inhibits the proliferation of B and T cells (Halloran, 1996). MMF, as with
70 most immunosuppressive drugs, was developed to prevent organ rejection following
71 transplantation. However, MMF is also currently prescribed for the treatment of lupus as a
72 steroid sparing drug (Kapitsinou et al., 2004). MMF is known to have reduced toxicity when
73 compared to CyP treatment (Mok, 2016) and may be able to replicate the leukopenia that is
74 necessary for SNV disease in hamsters (Brocato et al., 2014). Therefore, we also investigated the
75 use of orally-administered MMF as an immunosuppressant alone and in combination with the
76 steroid Dex in the SNV hamster disease model.

77 **RESULTS**

78 **SNV Tissue Burden**

79 We have previously demonstrated that hamsters immunosuppressed with Dex and CyP
80 and infected with SNV by the intramuscular (i.m.) route develop lethal HPS disease (Brocato et
81 al., 2014). To refine and expand this proof-of-concept work, a serial sacrifice study was
82 performed in which immunosuppressed hamsters were infected with 2,000 PFU SNV by the i.m.

83 route or 4,000 PFU SNV by the intranasal (i.n.) route and then viral and immune response
84 kinetics were analyzed every two days. The kinetics of SNV in lung, kidney, liver, spleen, and
85 heart were determined by viral genome detection (**Fig. 1A,B**). In each of the organs tested, there
86 was approximately a 6-8 day lag in SNV genome detection from hamsters infected by the i.n.
87 route when compared to i.m. challenge (**Fig. 1C**).

88 We have previously reported on the pathology in lungs from immunosuppressed hamsters
89 infected with SNV (Brocato et al., 2014). Here, we expand these pathology findings to the liver,
90 kidney, spleen, and heart. Despite high viral load in these assayed organs, no significant lesions
91 were observed by histology in liver or kidney at any timepoint (**Table S1**). Neutrophilic
92 granulocytosis was observed in the red pulp of the spleens from immunosuppressed infected and
93 uninfected hamsters on day 6 in SNV i.m. hamsters and days 6 and 8 on SNV i.n. hamsters. In
94 addition, mild myocardial degeneration and necrosis was observed in the heart of 8% of infected
95 and uninfected hamsters; this lesion was not specific to treatment group or day postinfection and
96 likely represents a commonly described background lesion in hamsters (McInnes, 2012). Neither
97 of these observed lesions were specifically colocalized with SNV antigen. Positive staining for
98 SNV antigen was detected in centrilobular hepatocytes and Kupffer cells in liver tissue sections
99 (**Fig. 2A**). Similarly, glomerular mesangial cells were positive for SNV antigen in kidney tissue
100 sections (**Fig. 2B**), macrophages and fibroblastic reticular cells were positive in the red pulp of
101 the spleen (**Fig. 2C**), and cardiac myocytes and capillary endothelial cells were positive in heart
102 tissue sections (**Fig. 2D**). This observed positive staining was present in animals from both
103 challenge routes.

104 The serial pathology data indicate that, regardless of challenge route, the lung is the
105 major site of virus replication. The expression of proinflammatory and immunomodulatory
106 cytokine-related genes and transcription factors were analyzed in lung tissue from
107 immunosuppressed, SNV-infected (i.m.) hamsters (**Fig. 3A,B,C**). Day 0 represents data points
108 from immunosuppressed, uninfected controls. Increased expression of the proinflammatory
109 cytokines IL1 β and IL6, and VEGF genes were observed later in infection, and coincided with
110 viremia in the lung (**Fig. 1A**). Increased expression of IRF2, STAT2, and iNOS were detected
111 throughout viral infection. Expression of the IFN-stimulated genes (ISGs) protein kinase R
112 (PKR), oligoadenylate synthetase 3 (OAS3), and IFN-induced GTP-binding protein (Mx2) were

113 slightly elevated at various times through the acute infection. Increased levels of expression of
114 IFN γ and TNF α were not detected in immunosuppressed, SNV-infected hamsters. Levels of
115 serum IFN- β were evaluated in immunocompetent and immunosuppressed hamsters infected
116 with SNV. There was a significant reduction in IFN- β expression in infected hamsters treated
117 with Dex and CyP 2 days postinfection ($p<0.0001$). However, IFN- β expression levels between
118 untreated or Dex/CyP-treated animals were not statistically significant by days 4 and 6
119 postinfection (**Fig. 3D**).

120 **Optimization of SNV/Hamster Disease Models**

121 To evaluate the length of immunosuppression required for SNV-induced HPS, hamsters
122 were pretreated with a combination of Dex and CyP beginning on day -3 and
123 immunosuppression with Dex and CyP was stopped at various time points postinfection. On day
124 0, hamsters were challenged with 2,000 PFU by the i.m. route. Immunosuppression through day
125 7 postinfection resulted in significant lethality (day 7, $p<0.0001$, day 10, $p=0.0024$, day 13,
126 $p=0.0085$) (**Fig. 4A**). In contrast, immunosuppression through day 5 postinfection did not
127 produce uniform disease, but did result in a statistically significant 63% lethality ($p=0.0133$)
128 compared to untreated, infected hamsters. In surviving hamsters, day 28 (end of study) lung
129 tissue was evaluated for SNV genome by RT-PCR. Hamsters treated with Dex and CyP had
130 elevated levels of SNV present in the lungs when compared to untreated, infected controls (**Fig.**
131 **4B**).

132 Using immunosuppression beginning on day -3 through day 7 postinfection, a 50% lethal
133 dose (LD50) experiment was performed to determine the SNV dose necessary for hamsters to
134 develop HPS. As little as 2 PFU SNV resulted in lethal disease in 50% of the animals in that
135 group (**Fig. 4C**) when hamsters were challenged by the i.m. route. Statistical analysis determined
136 that the LD50 dosage is 2.5 PFU for this model. Hamster groups challenged with 20, 200, and
137 2,000 PFU SNV all exhibited statistically significant increases in lethality when compared to
138 uninfected hamsters ($p=0.0243$, $p=0.0020$, and $p=0.0020$, respectively). Lung tissue collected on
139 day 28 from surviving animals demonstrate the presence of SNV genome in treated, infected
140 hamsters (**Fig. 4D**).

141 For the SNV i.n. model, the length of immunosuppression with Dex and CyP was
142 determined by truncating immunosuppression to 13 and 16 days postinfection. When challenged
143 with 4,000 PFU SNV, only 12.5% of hamsters receiving Dex and CyP through day 13 developed
144 lethal disease. However, 87.5% of hamsters receiving Dex and CyP through day 16 developed
145 lethal disease resulting in a statistically significant reduction in survival ($p=0.0012$) (**Fig. 5A**). Of
146 the 7 surviving hamsters on day 28 (end of study), results of a nucleocapsid (N)-ELISA assay
147 indicate that 6 of the hamsters immunosuppressed to day 13 developed an antibody response to
148 SNV and the lone survivor from the Dex/CyP treatment through day 16 did not develop an
149 antibody response to SNV above the limit of detection for the assay (**Fig. 5B**). Hamsters that
150 developed lethal disease had approximately 10^5 molecules of small (S)-segment genome in the
151 lung (**Fig. 5C**). Hamsters surviving to day 28 had comparable levels of S-segment genome in
152 lung tissue; however, hamsters not receiving Dex and CyP developed an increased antibody
153 response to SNV infection when compared to hamsters receiving Dex and CyP through day 13,
154 although not statistically significant. There is not a direct correlation between the S-segment
155 genome detected and the magnitude of the antibody response as measured by N-ELISA.
156 However, the results of this experiment do support the hypothesis that longer
157 immunosuppression, through day 16 postinfection, is required to allow i.n. instilled SNV to
158 develop pathogenic disease in hamsters.

159 **Alternative Immunosuppression Approach**

160 The impact of replacing CyP with MMF was evaluated in SNV infection in hamsters.
161 Hamsters were immunosuppressed with either a combination of Dex and CyP, CyP alone, a
162 combination of Dex and MMF, MMF alone, or administered no immunosuppressive treatment
163 beginning on day -3 prior to infection through day 10 postinfection. All hamsters treated with
164 MMF were administered a dosage of 30 mg/kg/day. Immunosuppression with the combination of
165 Dex and CyP provided the greatest lethality (86%, $p=0.0029$ when compared to no treatment
166 controls) (**Fig. 6A**), followed by immunosuppression with CyP alone (57%, $p=0.0346$ when
167 compared to no treatment controls) thereby confirming our previous report (Brocato et al., 2014).
168 Immunosuppression with the combination of Dex and MMF or MMF alone resulted in 12% and
169 0% lethality, respectively. In addition, immunosuppression using solely MMF did not reduce
170 WBCs (**Fig. 6B**). Lung tissue collected from a subset of animals on day 12 postinfection

171 demonstrates that Dex administered in combination with either CyP or MMF is required for a
172 statistically significant increase in SNV genome over No Treatment controls ($p=0.0219$ and
173 $p=0.0089$, respectively) (**Fig. 6C**). Correspondingly, the Dex/CyP and Dex/MMF groups had
174 notable interstitial inflammation in the day 12 lung tissue (3/3 hamsters in both groups), more so
175 than groups receiving CyP or MMF alone, or left untreated (2/3, 2/3, and 0/3 hamsters per group,
176 respectively) (data not shown). RNA analysis of lung homogenates collected from surviving
177 animals on day 28 postinfection also supports the requirement for including Dex in a
178 combination treatment (**Fig. 6D**). Furthermore, results of an N-ELISA conducted with sera from
179 surviving hamsters on day 28 postinfection show that the ability of hamsters to develop SNV-
180 specific antibodies is reduced in hamsters immunosuppressed with CyP (alone [$p=0.0179$ when
181 compared to no treatment controls] or in combination with Dex) but not MMF (alone or in
182 combination with Dex) (**Fig. 6E**).

183 Increased dosages of MMF were evaluated in uninfected hamsters to determine if a
184 higher concentration could reduce WBCs to levels observed in Dex/CyP treatment. Hamsters
185 were treated with MMF at concentrations ranging from 30-360 mg/kg for two days by the oral
186 route. On the third day, hematology analysis was conducted. The results of this dosing study
187 indicate that even a >10-fold increase in MMF was not able to significantly reduce WBCs when
188 compared to no treatment controls (**Fig. 7**).

189 DISCUSSION

190 Animal models are an essential component for elucidating viral pathogenesis, testing and
191 evaluating vaccines, antivirals, and biologicals, and the potential licensing of those products. Our
192 first report on the SNV/immunosuppressed hamster model focused mainly on the disease in the
193 target organ (i.e. lung) (Brocato et al., 2014); in the current study we demonstrate that SNV
194 disseminates to other organs, namely the kidney, liver, spleen, and heart. Others have
195 demonstrated that dissemination can occur after serially passaging SNV through
196 immunocompetent Syrian hamsters (Safronetz et al., 2013b); here we demonstrate that this
197 dissemination can be replicated with low-passage cell culture virus in immunosuppressed
198 hamsters. Similar to immunocompetent hamsters infected by Andes virus (ANDV) by the i.m.
199 route (Wahl-Jensen et al., 2007), there is an approximate 6 day incubation period before viremia
200 can be detected in immunosuppressed hamsters infected with SNV by the i.m. route. A 6-8 day

201 delay in viremia is observed when comparing the SNV/immunosuppressed i.m. and i.n. routes of
202 infection. A similar delay in death is observed between these two routes for ANDV infection in
203 hamsters (Hooper et al., 2008). These ANDV/hamster models have been used to demonstrate
204 that antivirals such as ribavirin, favipiravir, and neutralizing antibodies are only efficacious if
205 administered prior to the detection of viremia (Haese et al., 2015; Hooper et al., 2014; Hooper et
206 al., 2008; Ogg et al., 2013; Safronetz et al., 2013a). Efforts to expand this therapeutic window
207 have been largely unsuccessful. The SNV/immunosuppressed hamster model provides an
208 alternative small animal model for the evaluation of similar candidate medical countermeasures
209 against another important virus that causes HPS.

210 Viruses have evolved multiple mechanisms for subverting the host immune response
211 (Rouse and Horohov, 1986). ANDV modulation of early host innate responses both *in vivo*
212 (Safronetz et al., 2011) and *in vitro* (Levine et al., 2010) may contribute to the establishment of
213 the infection and the associated pathogenicity observed in the hamster model. The lack of disease
214 associated with SNV infection in immunocompetent hamsters may indicate that, unlike ANDV,
215 SNV is unable to modulate early innate responses. We hypothesize that dissemination of SNV
216 and pathogenicity in immunosuppressed hamsters is caused by modulation of early host innate
217 responses and a subsequent inability of the adaptive immune response to contain and clear
218 infection. We further speculate that the timing of the immune response to virus infection is
219 critical to result in an asymptomatic infection or death of the hamster. Negative regulation of
220 transcription factors AP-1 and NF- κ B by Dex treatment suppresses cytokines and chemokines
221 such as IL-2, IL-6, IFN- γ , and IL-8 (Karin, 1998). In addition, glucocorticoid treatment has been
222 shown to inhibit signaling by cytokines that utilize the Jak-STAT pathway, namely IL-2 and IL-
223 12 (Hu et al., 2003). In the current study, hamsters treated with Dex did not have early increased
224 expression of these cytokines and chemokines. Expression of IL-6 later in infection is also
225 observed in human HPS cases (Borges et al., 2008; Morzunov et al., 2015) and represents the
226 hamsters' ability to initiate a proinflammatory response despite Dex treatment. VEGF and IL-6
227 promote migration of mononuclear cells to the lung and these cells are observed in the
228 histological analysis of lung sections from SNV-infected, immunosuppressed hamsters (Brocato
229 et al., 2014). The lack of STAT1 upregulation, impacted by Dex treatment (Bhattacharyya et al.,
230 2011), contributes to the suppression of IFN- γ (Hu et al., 2003), similar to the expression levels
231 of STAT1 and IFN- γ presented herein. Similarly, a reduction in IFN- β expression at short

232 timepoints after SNV challenge (i.e. 2 days) likely contributes to increased SNV dissemination
233 and pathogenicity in immunosuppressed hamsters compared to immunocompetent hamsters. The
234 ANDV N has been shown to inhibit type I IFN signaling responses whereas SNV N does not
235 (Cimica et al., 2014). Treatment of hamsters with Dex may allow SNV to replicate in this host in
236 a manner similar to ANDV infection of immunocompetent hamsters.

237 SNV readily infects hamsters with a 50% infectious dose (ID50) of less than 2 PFU
238 (Hooper et al., 2001). When hamsters are immunosuppressed from day -3 to 7 days postinfection
239 and infected with SNV by the i.m. route, an LD50 dosage of 2.5 PFU was calculated. Thus, the
240 ability to infect hamsters appears to be very efficient regardless of whether the hamster is
241 immunosuppressed or not. These optimization experiments demonstrate the balance of the length
242 of immunosuppression required and challenge dose for a uniformly lethal model for the i.m. and
243 i.n. routes of exposure.

244 MMF has recently been accepted as a treatment to patients suffering from lupus nephritis,
245 as an alternative to CyP or CyP combined with a glucocorticoid (Tesar, 2016). By replacing CyP
246 with MMF in the SNV/hamster model, we evaluated the steroid sparing potential of MMF. This
247 experiment was rationalized by the possibility of administering a single drug, and the ability to
248 compound this drug into the feed for future experiments. A hallmark of the Dex/CyP model is
249 pronounced leukopenia resulting in a diminution of adaptive and innate immune responses that
250 allow SNV to replicate and cause acute disease. However, hamsters treated with MMF alone did
251 not exhibit the reduction in WBCs observed with CyP treatment, nor allow SNV to replicate in
252 the lung to levels observed when Dex was incorporated in the treatment regimen.

253 Immunosuppressive drugs were administered through day 10 postinfection. This timepoint was
254 selected from the truncated immunosuppression experiment (**Fig. 4A**) demonstrating that
255 Dex/CyP treatment through day 10 resulted in 100% lethality. A single death was observed in the
256 Dex/MMF treatment group leading us to hypothesize that potentially extending the treatment
257 regimen beyond day 10 may have resulted in increased lethality. Increased concentrations of
258 MMF did not reduce WBCs in treated hamsters (**Fig. 7**); whether this is a species-specific
259 phenomenon or a higher dosage of MMF is needed to induce leukopenia is currently unknown.
260 Future efforts will utilize the combination of Dex and CyP in the SNV/hamster model.

261 The SNV/immunosuppressed hamster model represents an alternative small animal lethal
262 disease model that recapitulates many of the salient features of the ANDV/hamster model and
263 human HPS disease. Further refinement of this model allows the testing of vaccines and medical
264 countermeasures to combat hantavirus disease and may provide an alternative animal model for
265 licensure of products under the FDAs “Animal Rule.”

266 **MATERIALS AND METHODS**

267 **Viruses, cells, and medium.** SNV strain CC107 (Schmaljohn et al., 1995) was propagated in
268 Vero E6 cells (Vero C1008, ATCC CRL 1586). Preparation of twice plaque-purified SNV stock
269 has been described previously (Hooper et al., 2001). Cells were maintained in Eagle’s minimum
270 essential medium with Earle’s salts containing 10% fetal bovine serum, 10 mM HEPES, pH 7.4,
271 and Penicillin Streptomycin (Invitrogen) at 1X, and gentamicin sulfate (50 µg/ml) at 37°C in a
272 5% CO₂ incubator.

273 **Dex, CyP, and MMF administration.** Water soluble Dex and CyP monohydrate were
274 purchased from Sigma-Aldrich. MMF was purchased from Selleck Chemicals. On the indicated
275 days, anesthetized hamsters were injected intraperitoneally (i.p.) with the indicated dosages per
276 body weight of drug diluted in sterile phosphate-buffered saline (PBS), pH 7.4. Hamsters were
277 treated with a loading dose of 16 mg/kg Dex and 140 mg/kg CyP on day -3, 8 mg/kg Dex on day
278 -2, 8 mg/kg Dex and 100 mg/kg CyP on day -1, 4 mg/kg Dex on days 0, 2, 3, 5, 6, 8, 9, 11, 12,
279 13, 15, 16, 17, 19, 20, 21, and 4 mg/kg Dex and 100 mg/kg CyP on days 1, 4, 7, 10, 14, 18, and
280 22. Each experiment specifies the length of Dex and CyP immunosuppression administered to
281 animals. Hamsters were administered 30 mg/kg MMF by the oral route daily from day -3 to day
282 10 postinfection (**Fig. 6**). Hamsters were administered 30-360 mg/kg MMF by the oral route for
283 2 days prior to hematology (**Fig. 7**).

284 **Challenge with hantavirus.** Female Syrian hamsters 6-8 wks of age and > 100g (Envigo,
285 Indianapolis, IN) were anesthetized by inhalation of vaporized isoflurane using an IMPAC 6
286 veterinary anesthesia machine. Once anesthetized, hamsters were injected with the indicated
287 concentration of virus diluted in PBS. Intramuscular (i.m.) (caudal thigh) injections consisted of
288 0.2ml delivered with a 1ml syringe with a 25-gauge, 5/8in needle. Intranasal (i.n.) instillation
289 consisted of 50µl total volume delivered as 25µl per nare with a plastic pipette tip. Groups of 8

290 hamsters were typically used for experimental treatments, unless otherwise stated. All work
291 involving hamsters was performed in an animal biosafety level 4 (ABSL-4) laboratory.
292 Euthanasia was performed on animals meeting early endpoint criteria.

293 **ELISA.** The enzyme-linked immunosorbent assay (ELISA) used to detect N-specific antibodies
294 (N-ELISA) was described previously (Elgh et al., 1997; Hooper et al., 1999). The endpoint titer
295 was determined as the highest dilution that had an optical density (OD) greater than the mean
296 OD for serum samples from negative-control wells plus 3 standard deviations. The Puumala N
297 antigen was used to detect SNV N-specific antibodies as previously reported (Xiao et al., 1993).
298 Hamster-specific IFN- β ELISA (MyBiosource, San Diego, CA) was run according to
299 manufacturer's published protocols.

300 **Isolation of RNA and real-time PCR.** Approximately 250 mg of organ tissue was homogenized
301 in 1.0 ml TRIzol reagent using gentleMACS M tubes and a gentleMACS dissociator on the RNA
302 setting. RNA was extracted from TRIzol samples as recommended by the manufacturer. The
303 concentration of the extracted RNA was determined using a NanoDrop 8000 instrument and
304 raised to a final concentration of 10 ng/ μ l. Real-time PCR was conducted on a BioRad CFX
305 thermal cycler using an Invitrogen Power SYBR Green RNA-to-Ct one-step kit according to the
306 manufacturer's protocols. Primer sequences are SNV S 26F 5'-CTA CGA CTA AAG CTG
307 GAA TGA GC-3' and SNV S 96R 5'-GAG TTG TTG TTC GTG GAG AGT G-3" (Trombley et
308 al., 2010). Cycling conditions were 30 min at 48°C, 10 min at 95°C, followed by 40 cycles of 15
309 sec at 95°C and 1 min at 60°C. Data acquisition occurs following the annealing step.

310 Host responses were monitored using hamster-specific primers for IL1 β , IL2, IL6, IL10,
311 IL12p35, IRF1, IRF2, IFN γ , iNOS, Mx2, OAS3, PKR, STAT1, STAT2, TNF α , and VEGF (Toth
312 et al., 2015; Zivcec et al., 2011) using HPRT as an internal control. Data was analyzed using the
313 $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001) where the Ct was first normalized to the internal
314 control and then compared to an immunosuppressed, mock-treated animal.

315 **Hematology.** Blood samples collected in lithium heparin capillary blood collection tubes were
316 analyzed using an Advia 120 hematology analyzer using proprietary software version 3.1.8.0-
317 MS. The dog setting was used for the complete blood count and guinea pig setting was used for
318 the differential.

319 **Preparation of tissues for histology.** Tissues were fixed in 10% neutral-buffered formalin for
320 ≥ 21 days. Tissues were then trimmed, processed under vacuum through increasing
321 concentrations of alcohols, and embedded in paraffin. 5-6 μ m sections of paraffin embedded
322 tissue were cut and mounted on glass slides, stained with hematoxylin-eosin (H&E), and
323 mounted under a glass coverslip for histologic evaluation. Immunolocalization of SNV in tissues
324 was performed with an immunoperoxidase procedure (horseradish peroxidase EnVision system;
325 Dako) according to the manufacturer's directions. The primary antibody was an α -SNV
326 nucleocapsid rabbit polyclonal antibody diluted 1:3,000 (provided by Diagnostic Service
327 Division, USAMRIID). Negative controls included naïve hamster tissue incubated with
328 nonimmune rabbit IgG in place of the primary antibody and naïve hamster tissue exposed to the
329 primary antibody and negative serum. After deparaffinization and peroxidase blocking, tissue
330 sections were pretreated with proteinase K for 6 min at room temperature, rinsed, and then
331 covered with primary antibody and incubated at room temperature for 1 hr. They were rinsed,
332 and then the peroxidase-labeled polymer (secondary antibody) was applied for 30 min. Slides
333 were rinsed, and a substrate-chromogen solution (3,3'-diaminobenzidine; Dako) was applied for
334 5 min. The substrate-chromogen solution was rinsed off the slides, and the slides were stained
335 with hematoxylin and rinsed. The sections were dehydrated and cleared with xyless, and then a
336 coverslip was placed.

337 **Statistical analysis.** Survival curves were compared with Kaplan-Meier survival analysis with
338 log-rank comparisons and Dunnett correction. A Bayesian probit model was used to estimate
339 95% highest posterior density intervals for a 50% lethal dose calculation. Comparison of viral
340 genome results was done using a one-way ANOVA with Dunnett's multiple comparison test.
341 Comparison of WBC was done using a paired t test. Comparison of N-ELISA was done using
342 Mann-Whitney test. *P* values of less than 0.05 were considered significant. Analyses were
343 conducted using GraphPad Prism (version 6); Bayesian analyses were performed using Stan
344 2.1.0.

345 **Ethics statement.** Animal research was conducted under an IACUC approved protocol at
346 USAMRIID (USDA Registration Number 51-F-00211728 & OLAW Assurance number A3473-
347 01) in compliance with the Animal Welfare Act and other federal statutes and regulations
348 relating to animals and experiments involving animals. The facility where this research was

349 conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory
350 Animal Care, International and adheres to principles stated in the Guide for the Care and Use of
351 Laboratory Animals, National Research Council, 2011.

352 **FIGURE LEGENDS**

353 **Figure 1. SNV kinetics in Syrian hamster organs.** Three hamsters from each of the
354 immunosuppressed, SNV i.m. and i.n. groups were euthanized at each timepoint. Tissues were
355 excised, homogenized, and RNA isolated for SNV genome detection by RT-PCR. SNV S-
356 segment kinetics was measured in lungs, kidneys, livers, spleens, and hearts in hamsters infected
357 by the **A**) i.m. route and **B**) i.n. route. Mean values and \pm SD are shown. **C**) SNV genome organ
358 burden is shown as the mean of all organs evaluated from all animals at each timepoint. The lag
359 in SNV genome organ burden between the two routes of infection is shown as the blue arrow. A
360 single hamster on day 12 from the Dex/CyP SNV i.n. group is shown separately as an outlier
361 depicted by the filled triangle (\blacktriangle).

362 **Figure 2. SNV antigen detection in hamster organs by immunohistochemistry.**
363 Immunohistochemistry using an α -nucleocapsid antibody was performed on **A**) liver tissue, **B**)
364 kidney tissue, **C**) spleen tissue, and **D**) heart tissue from immunosuppressed, SNV-infected
365 hamsters collected on either day 10 (liver, kidney, and heart) or day 20 (spleen) postinfection. **A**,
366 **B**, **C**, 400X magnification. **D**, 200X magnification. Size bars are indicated in each panel.

367 **Figure 3. Normalized fold expression of select cytokines in lung tissue of**
368 **immunosuppressed, SNV-infected hamsters. A, B, C)** RNA from homogenized lung tissue
369 was isolated from immunosuppressed, SNV i.m. hamsters (n=3 per timepoint). Gene regulation
370 was normalized to the reference gene HPRT and compared using the $\Delta\Delta$ Ct method on the
371 indicated days. Individual values are shown with the horizontal line representing the mean. **D)**
372 Serum IFN- β levels from SNV-infected and immunosuppressed, SNV-infected hamsters
373 analyzed by ELISA. ***, $P < 0.001$; ns, not significant.

374 **Figure 4. Optimization of immunosuppressed SNV i.m. model. A)** The length of
375 immunosuppression with Dex and CyP required for the development of lethal HPS was
376 determined in groups of 7 or 8 hamsters each. Hamsters were challenged with 2,000 PFU i.m.
377 and immunosuppression beginning on day -3 was truncated to days 5, 7, 10, and 13 days

378 postinfection. Hamsters were monitored for survival. **B)** Lung tissues collected from surviving
379 hamsters (day 28) were analyzed for the presence of SNV genome by RT-PCR. **C)** Groups of 10
380 hamsters each were immunosuppressed with Dex and CyP from day -3 through day 7
381 postinfection. Hamsters were challenged with the indicated concentration of virus on day 0 and
382 monitored for survival. **D)** Lung tissues collected from surviving hamsters (day 28) were
383 analyzed for the presence of SNV genome by RT-PCR. *, P < 0.05; **, P < 0.01; ***, P <
384 0.001.

385 **Figure 5. Optimization of immunosuppressed SNV i.n. model.** **A)** Groups of 8 hamsters each
386 were immunosuppressed with Dex and CyP beginning on day -3 through the indicated day. All
387 hamsters were infected with 4,000 PFU SNV by the i.n. route and monitored for survival. **B)**
388 Sera from surviving hamsters on day 35 were analyzed by N-ELISA. **C)** Lung tissues collected
389 after the onset of HPS (blue circles indicate hamsters were euthanized or found dead) or on day
390 35 postinfection were analyzed for the presence of SNV genome by RT-PCR. **, P < 0.01.

391 **Figure 6. Alternative immunosuppression of hamsters using MMF.** Groups of 6-8 hamsters
392 each were immunosuppressed with CyP, Dex and CyP, MMF, Dex and MMF, or left untreated
393 beginning on day -3 through day 10 postinfection. All hamsters were infected with 2,000 PFU
394 SNV by the i.m. route on day 0. Hamsters were monitored for **A)** survival and **B)** WBCs. **C)** A
395 subset of hamsters not used for survival were euthanized on day 12 and lung homogenates
396 analyzed for SNV genome by RT-PCR. Hamsters surviving to day 28 were euthanized and **D)**
397 lung homogenates analyzed for SNV genome by RT-PCR and **E)** serum analyzed by N-ELISA.
398 *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant.

399 **Figure 7. Dose optimization of MMF in hamsters.** Groups of 3 hamsters each were
400 administered increasing concentrations of MMF for two days by the oral route. Whole blood
401 collected on day 3 was analyzed for WBCs.

402

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410

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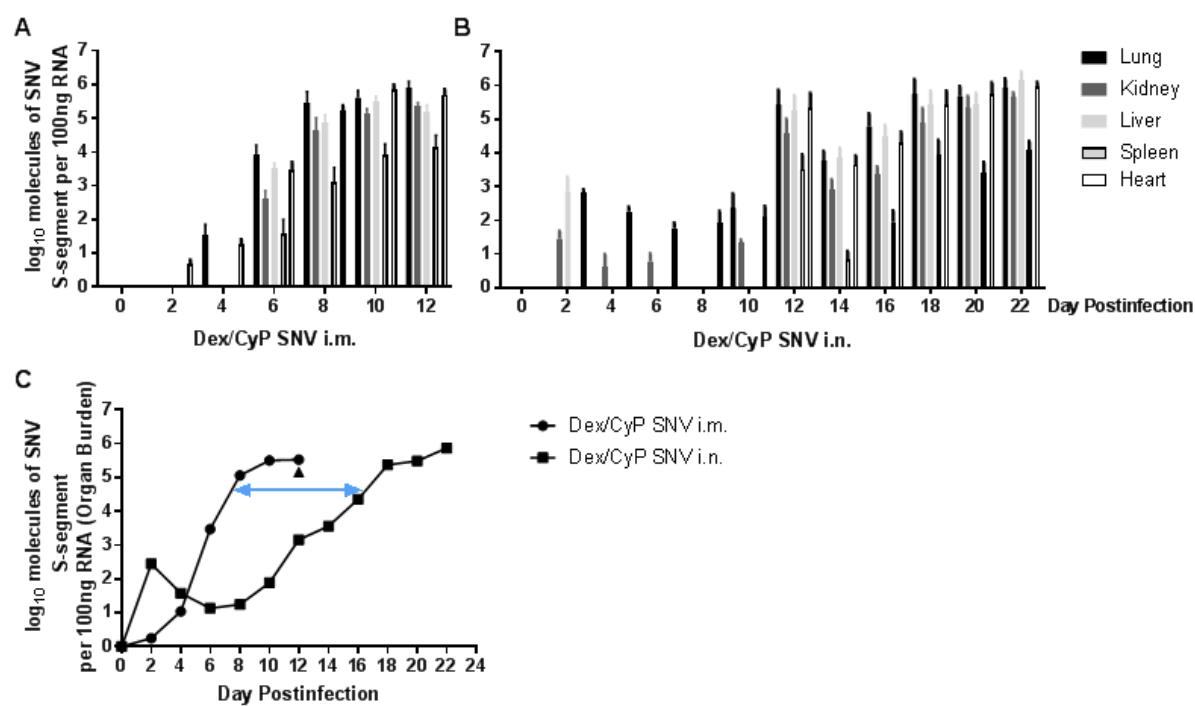
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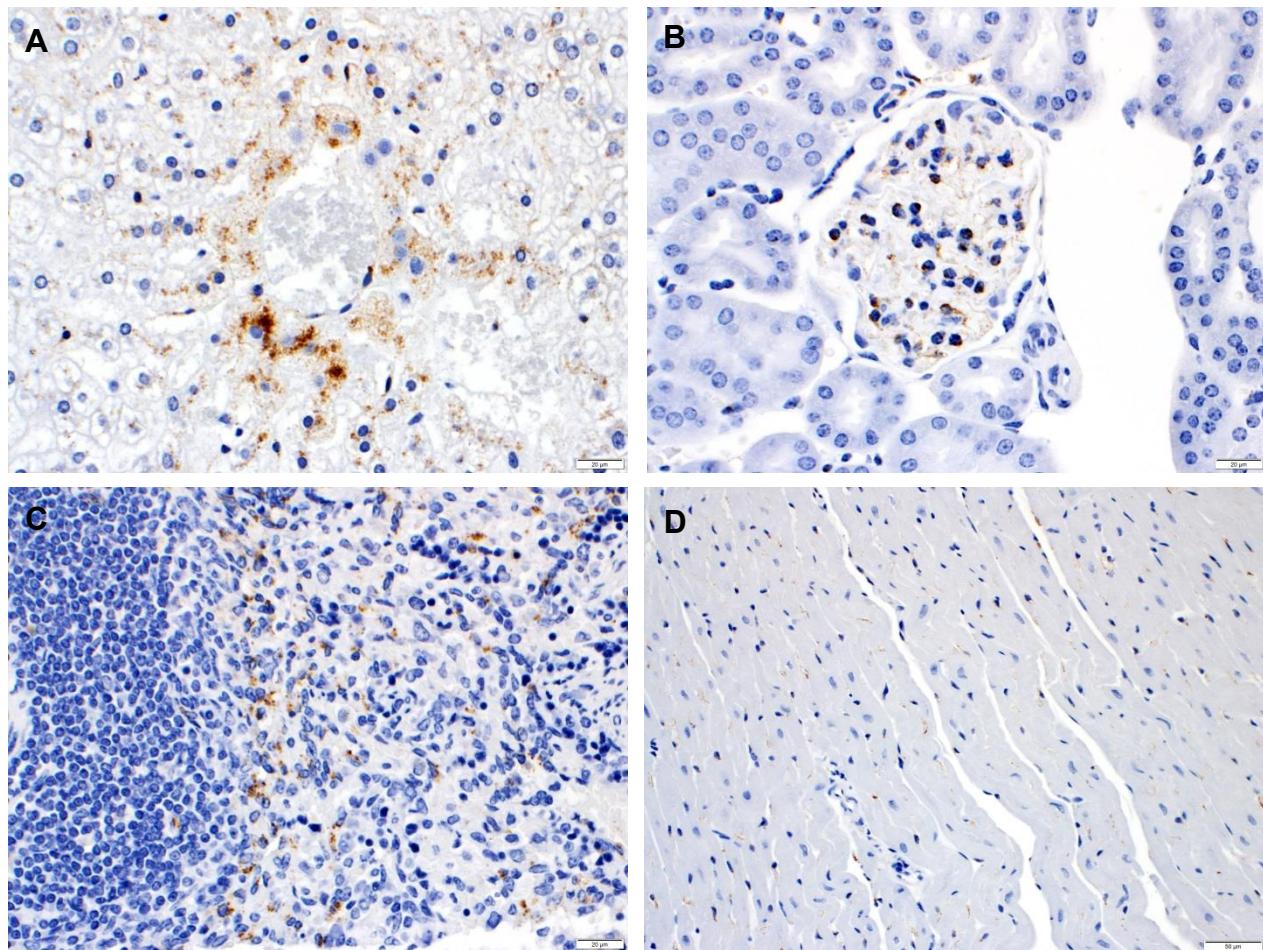
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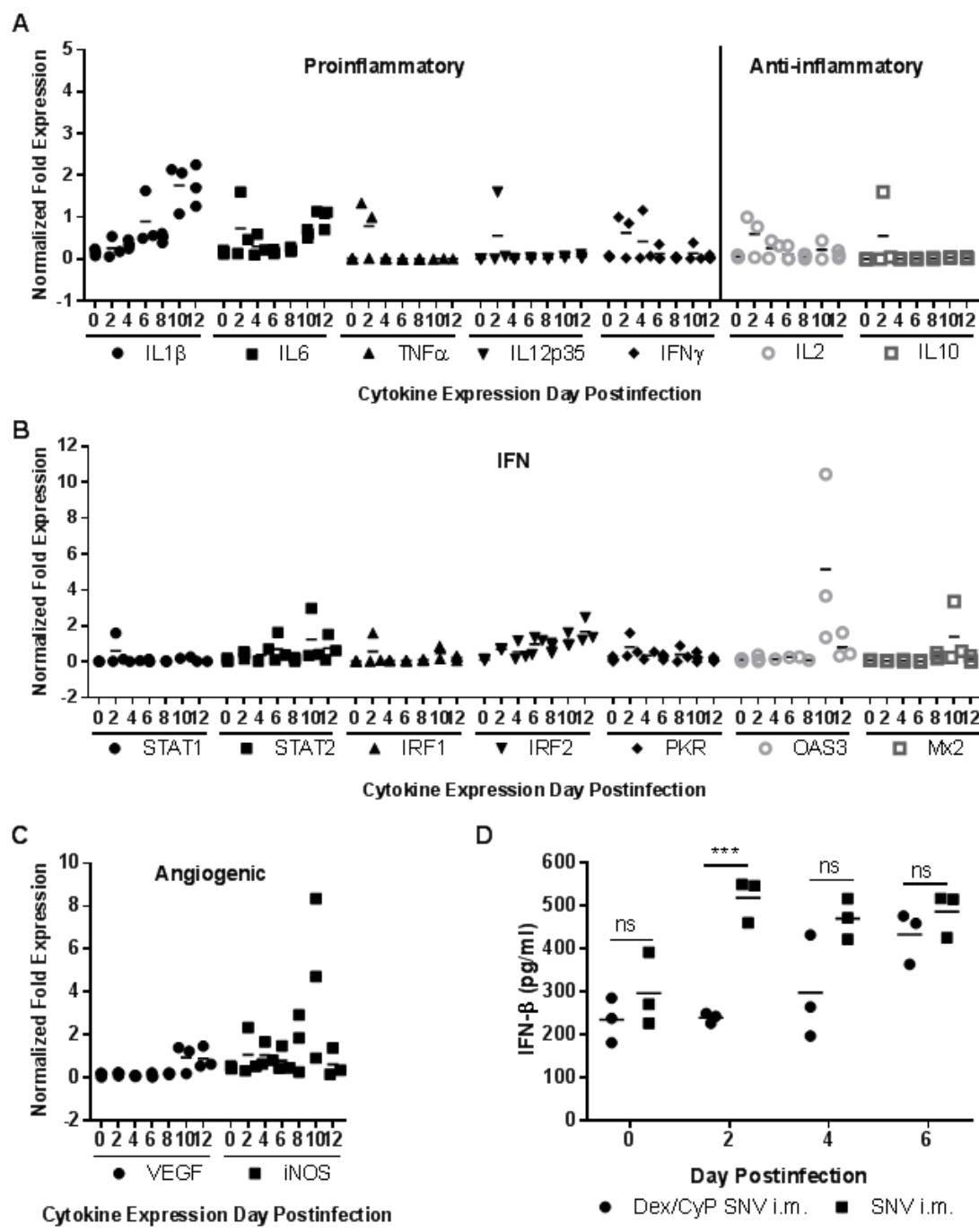
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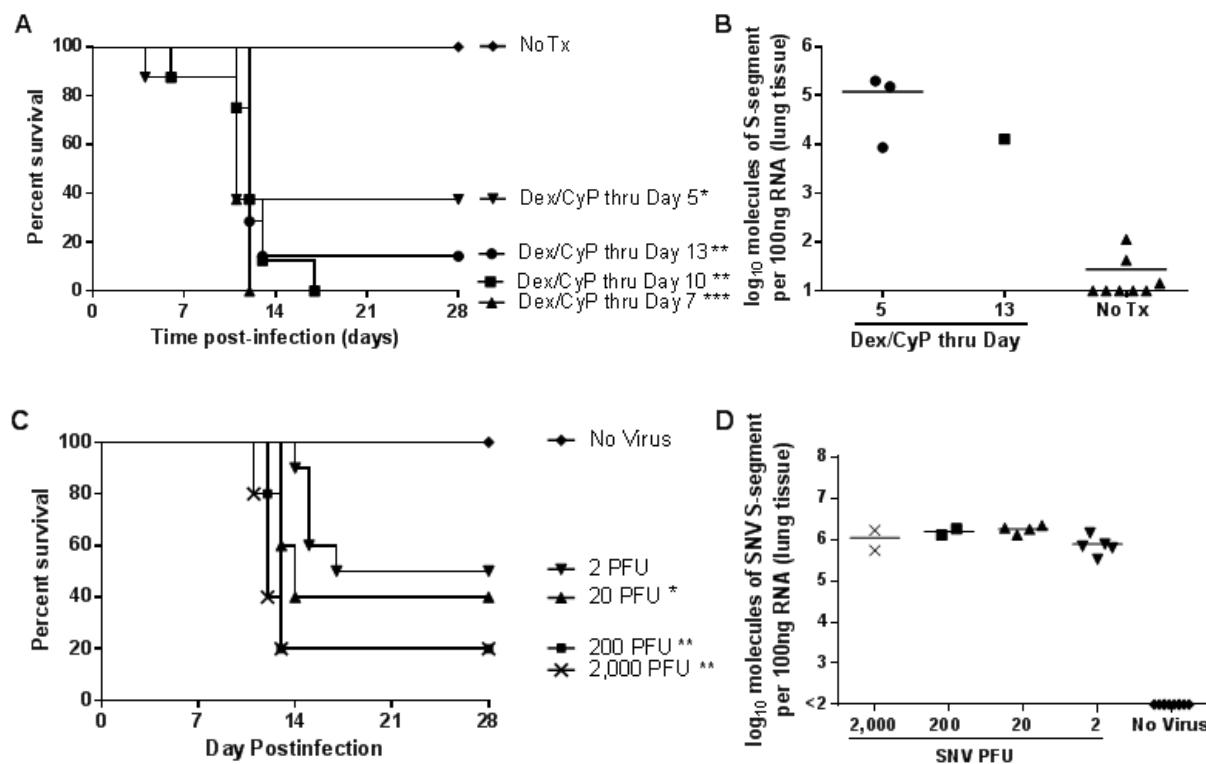
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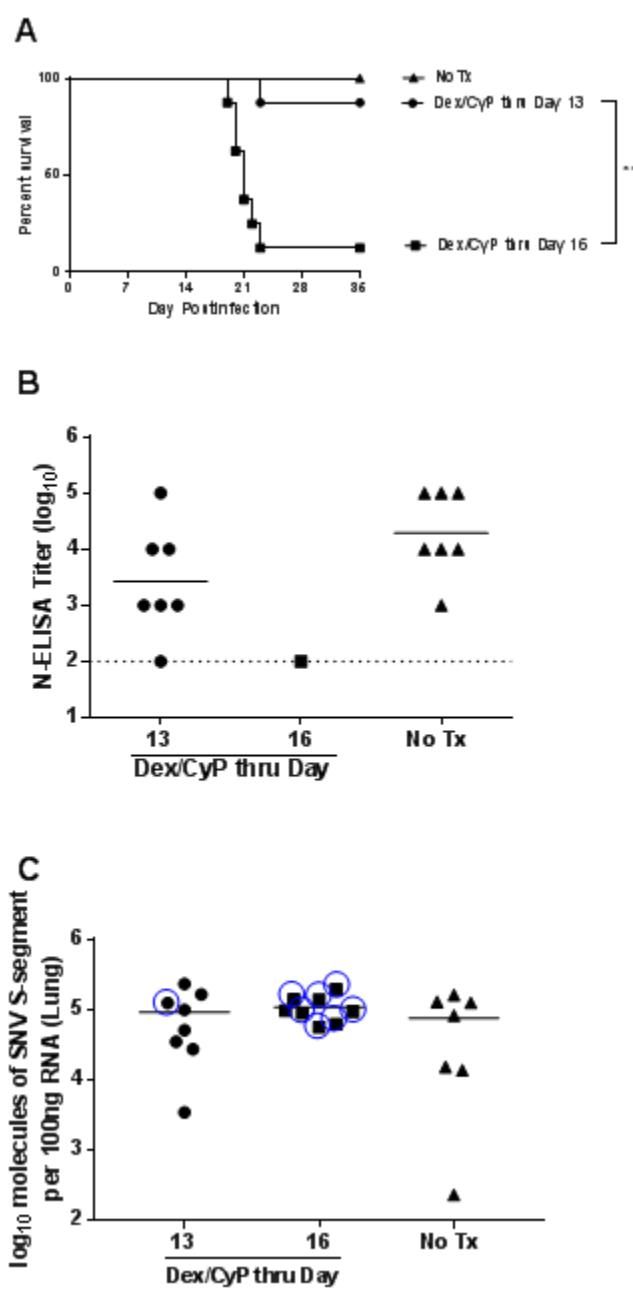
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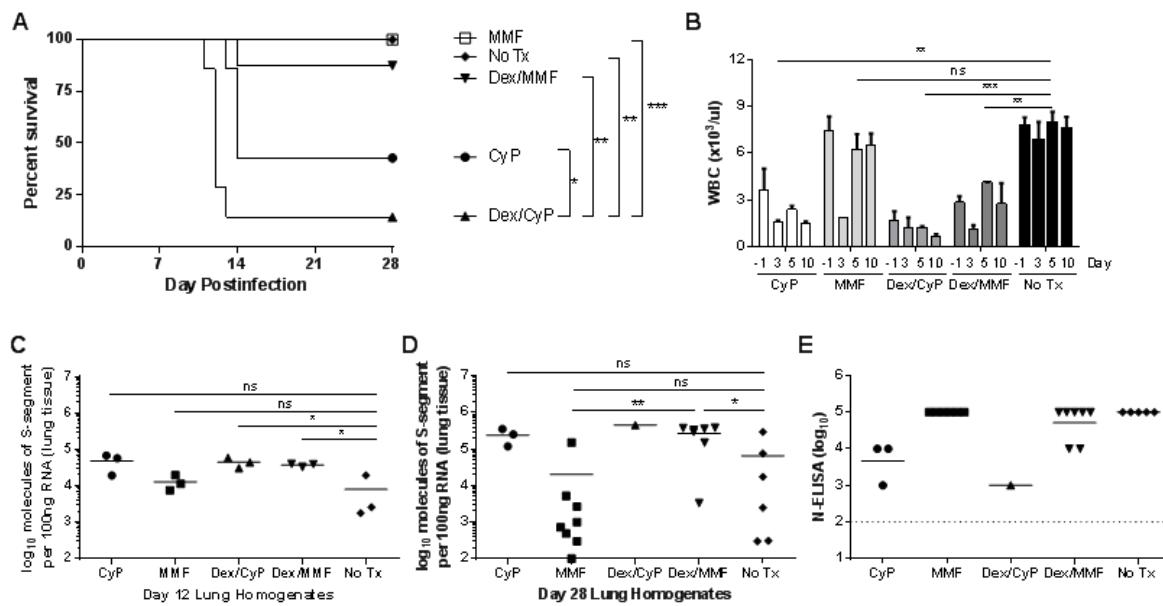
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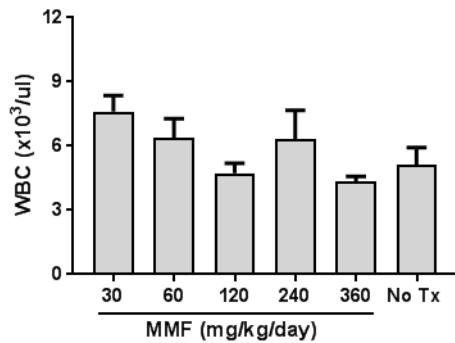


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547 Figure 7



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551 **Table S1.** Histologic findings in Dex/CyP SNV i.m., SNV i.n., and uninfected hamsters.

Dex/CyP SNV i.m.	Day Postinfection	Hamster Organ				
		Lung Histologic Finding: Interstitial Inflammation	Kidney Histologic Finding	Liver Histologic Finding	Spleen Histologic Finding: Red Pulp	Heart Histologic Finding: Myocardial Degeneration/Necrosis
	0	-	-	-	-	-
	2	-	-	-	-	+
	4	-	-	-	-	-
	6	++	-	-	++	-
	8	+	-	-	-	-
	10	++++	-	-	-	-
	12	++	-	-	-	-
<hr/>						
Dex/CyP SNV i.n.	Day Postinfection					
	0	-	-	-	-	-
	2	-	-	-	-	+
	4	-	-	-	-	+
	6	-	-	-	+	-
	8	+	-	-	+	-
	10	+	-	-	-	-
	12	+	-	-	-	-
	14	++	-	-	-	-
	16	++	-	-	-	++
	18	++++	-	-	-	-
	20	+++	-	-	-	-
	22	+++	-	-	-	-
<hr/>						
Dex/CyP No Virus	Day Postinfection					
	0	-	-	-	-	-
	2	-	-	-	-	++
	4	-	-	-	-	-
	6	+	-	-	-	+
	8	ND	-	-	+	-
	10	-	-	-	-	-
	12	+	-	-	+	-

552 Histopathological findings were scored as follows: +, very mild; ++, mild; +++, moderate; +++, marked; +++++, severe; -, negative or minimal finding. ND, no data due to autolysis of tissue.